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PCT / FI 2004 / 000540

Helsinki 29.9.2004

Rec'd PCT/PTO 19 APR 2005

E T U O I K E U S T O D I S T U S
P R I O R I T Y D O C U M E N T

REC'D 22 OCT 2004
WIPO PCT

Hakija Applicant	**FIT Biotech Oyj Plc** **Tampere**
Patenttihakemus nro Patent application no	**20031319**
Tekemispäivä Filing date	**15.09.2003**
Kansainvälinen luokka International class	**C12N**
Keksinnön nimitys Title of invention	**"Novel selection system"** **(Uusi valintasysteemi)**

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Osoite: Arkadiankatu 6 A
P.O.Box 1160
FIN-00101 Helsinki, FINLAND
Puhelin: 09 6939 500
Telephone: + 358 9 6939 500
Telefax: 09 6939 5328
Telefax: + 358 9 6939 5328

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Novel selection system

Field of the invention

The present invention relates to a novel selection system, which is based on the use of the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and to the use of a bacterial strain deficient of the *ara*D gene. The present invention further relates to novel vectors containing the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof and to novel bacterial strains deficient of the *ara*D gene. The present invention additionally relates to a method of selecting the cells transformed with a plasmid, which contains the gene of interest.

Background of the invention

An essential requirement for effective genetic engineering of bacteria and other cells propagated in cell cultures is the capacity to select the cells with a specific genotypic alteration. The most common selection strategy in recombinant DNA technology is to include a selection marker in the cloning vector or plasmid. A selection marker can be a cloned gene or a DNA sequence, which allows the separation of the host cells containing the selection marker from those not containing it. The selection marker together with a suitable selection medium maintains the cloning vector in the cells. Otherwise, since the replication of plasmids is an energetic burden for the bacterial host, in a growing culture the bacteria, which have lost the plasmid, would have a growth advantage over the cells with the plasmid.

For most purposes, an antibiotic resistance gene is a commonly used selection marker. However, for the production of recombinant therapeutics, where the goal is to generate a product, such as a DNA vaccine, in high yield for administration in patients, the use of antibiotic resistance genes presents problems: the spread of antibiotic resistant pathogens is a serious worldwide problem [Levy, S. B., J. Antimicrob. Chemother. 49 (2002) 25-30]. Therefore the antibiotic resistance genes cannot have extensive use in the pharmaceutical industry, and for instance, according to the regulations of the U.S. Food and Drug Administration, no antibiotic resistance genes are allowed in experimental DNA vaccines entering the third phase.

Alternatively, antibiotic-free selection systems have been suggested. Such antibiotic-free selection systems include bacterial toxin-antitoxin

systems [Engelberg-Kulka, H. and Glaser, G., Annu Rev Microbiol 53 (1999) 43-70], genes responsible for resistance against heavy metals, such as tellurium [Silver, S. and Phung, L. T., Annu Rev Microbiol 50 (1996) 753-789], and systems, in which the plasmid encodes a gene complementing a host auxotrophy [Wang, M.D., *et al.*, J. Bacteriol. 169 (1987) 5610-5614].

US Patent Application 2000/0014476 A1 generally discloses, inter alia, the use of a non-antibiotic selection marker, which may be a gene whose product is necessary for the metabolism of the cell under certain culturing conditions, such as a catabolism gene, which makes it possible for the cell to assimilate a certain substance present in the culture medium (specific carbon or nitrogen source) etc. No specific examples of such suitable genes are given. This approach is not necessarily applicable for commercial production, since the deletion an essential component, such as an amino acid or a carbon source, from the growth medium reduces the yield, which is not desirable. Additionally, the manipulation of the growth medium in terms of omitting an essential nutritient may considerably increase the cost of the growth medium, since commercially available nutritient mixtures must be replaced by individual nutritients.

For commercial therapeutic purposes it would be of advantage to use a gene, which is not essential for the growth of the host but whose manipulation still affects the growth in selected circumstances. Additionally, in view of the therapeutic use, it would be of advantage to use a gene, whose deletion leads to accumulation of compounds, which are toxic to the host cell but not toxic to mammalians, including humans. Also it would be of advantage to use smaller genes, which in turn would allow the construction of smaller plasmids for which the energy consumption for replication is smaller and thus the growth rate of bacterial culture and plasmid yield are improved.

Short description of the invention

The object of the present invention is to provide a novel antibiotic-free selection system, which avoids the problems of previously disclosed selection systems for use in the production of recombinant therapeutic products.

Another object of the invention is to provide a novel antibiotic-free selection system, which can be safely used in the production of recombinant therapeutic products in terms of the environment and the patient safety.

A further object of the invention is to provide a novel antibiotic-free selection system, which can be cost-effectively used in the production of recombinant therapeutic products using standard growth mediums.

A still further object of the invention is to provide a novel antibiotic-free selection system which provides an increased growth rate and improved yield.

Yet another object of the present invention is to provide a novel vector containing a selection marker, which is non-toxic to the environment and the humans and which is capable of a long-term maintenance in the host.

Yet another object of the present invention is to provide a novel host cell containing a gene defect, which is not hazardous to the environment.

Still another object of the present invention is to provide a method for selection of cells carrying a gene of interest for the production of recombinant therapeutic products.

It was surprisingly found that the objects of the present invention are met by the use of the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and the use of a specific bacterial host deficient of the *ara*D gene.

Accordingly, the present invention provides a novel selection system comprising a vector carrying the *ara*D gene or the L-ribulose-5-phosphate 4-epimerase (EC 5.1.3.4.) coding gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and a bacterial host, especially an *Escherichia coli* strain, deficient of the *ara*D gene.

The present invention further provides novel vectors, which contain the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker.

The present invention further provides novel bacterial strains, which are deficient of the *ara*D gene.

The present invention further provides a method of selecting the cells transformed with a plasmid, which contains 1) the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and 2) the gene of interest, the method comprising inserting said plasmid into the *ara*D deficient host cell and growing the cells in a growth medium containing arabinose.

Drawings

Figure 1 shows the use of arabinose as a carbon source by the *E. coli* cells (Lin, 1987).

Figure 2 shows the map of S6wtd1EGFP. The coding sequences for the d1EGFP, E2 and kanamycin resistance marker aminoglycoside-3'-O-phosphotransferase (kana) are indicated by arrows. Additional features are indicated by solid boxes: 10E2BS – ten BPV E2 binding sites with high affinity; CMV-tk - human cytomegalovirus immediately early promoter and HSV Th gene leader sequence; intron – rabbit beta-globin gene intron with optimized SD and SA sites; tkpa – HSV Tk gene polyadenylation signal; RSV LTR – Rous sarcoma virus long terminal repeat; bgh pA – bovine growth hormone gene polyadenylation signal; pUCori – bacterial origin of replication derived from the pUC18 plasmid.

Figure 3 shows the map of S6wtd1EGFP*kana/ara*D1. The coding sequences for the d1EGFP, E2, kanamycin resistance marker aminoglycoside-3'-O-phosphotransferase (kana) and L-ribulose-5-phosphate-4-epimerase (araD) are indicated by arrows. Additional features are indicated by solid boxes: 10E2BS – ten BPV E2 binding sites with high affinity; CMV-tk - human cytomegalovirus immediately early promoter and HSV Th gene leader sequence; intron – rabbit beta-globin gene intron with optimized SD and SA sites; tkpa – HSV Tk gene polyadenylation signal; RSV LTR – Rous sarcoma virus long terminal repeat; bgh pA – bovine growth hormone gene polyadenylation signal; pUCori – bacterial origin of replication derived from the pUC18 plasmid.

Figure 4 shows the map of S6wtd1EGFP*kana/ara*D2. The coding sequences for the d1EGFP, E2, kanamycin resistance marker aminoglycoside-3'-O-phosphotransferase (kana) and L-ribulose-5-phosphate 4-epimerase (araD) are indicated by arrows. Additional features are indicated by solid boxes: 10E2BS – ten BPV E2 binding sites with high affinity; CMV-tk - human cytomegalovirus immediately early promoter and HSV Th gene leader sequence; intron – rabbit beta-globin gene intron with optimized SD and SA sites; tkpa – HSV Tk gene polyadenylation signal; RSV LTR – Rous sarcoma virus long terminal repeat; bgh pA – bovine growth hormone gene polyadenylation signal; pUCori – bacterial origin of replication derived from the pUC18 plasmid.

Figure 5 shows the map of S6wtd1EGFP/*ara*D1. The coding sequences for the d1EGFP, E2 and L-ribulose-5-phosphate 4-epimerase (araD) are indicated by arrows. Additional features are indicated by solid boxes: 10E2BS – ten BPV E2 binding sites with high affinity; CMV-tk - human cytomegalovirus immediately early promoter and HSV Th gene leader sequence; intron – rabbit beta-globin gene intron with optimized SD and SA sites; tkpa – HSV Tk gene polyadenylation signal; RSV LTR – Rous sarcoma virus long terminal repeat; bgh pA – bovine growth hormone gene polyadenylation signal; pUCori – bacterial origin of replication derived from the pUC18 plasmid.

Figure 6 shows the map of S6wtd1EGFP/*ara*D2. The coding sequences for the d1EGFP, E2 and L-ribulose-5-phosphate 4-epimerase (araD) are indicated by arrows. Additional features are indicated by solid boxes: 10E2BS – ten BPV E2 binding sites with high affinity; CMV-tk - human cytomegalovirus immediately early promoter and HSV Th gene leader sequence; intron – rabbit beta-globin gene intron with optimized SD and SA sites; tkpa – HSV Tk gene polyadenylation signal; RSV LTR – Rous sarcoma virus long terminal repeat; bgh pA – bovine growth hormone gene polyadenylation signal; pUCori – bacterial origin of replication derived from the pUC18 plasmid.

Figure 7A and 7B shows the electrophoretic analysis of the plasmid DNA of the S6wtd1EGFP/*ara*D1 (7A) and S6wtd1EGFP/*ara*D2 (7B) extracted from the E. coli strain AG1 delta *ara*D grown in different media.

Figure 8 shows the the restriction pattern analysis of the plasmid DNA of the S6wtd1EGFP/*ara*D1 and S6wtd1EGFP/*ara*D2 extracted from the E. coli strain AG1 delta *ara*D

Figure 9 shows the electrophoretic analysis of the S6wtd1EGFP/*ara*D2 in stability assay.

Figure 10A and 10B shows the restriction pattern analysis of the S6wtd1EGFP/*ara*D2 in stability assay.

Figure 11 shows the growth parameters of fed-batch fermentation of AG1Δ*ara*D S6wtd1EGFP/*ara*D2 measured and registered during fermentation. The abbreviations are as follows: sPump = feeding speed; pO2 = the oxygen concentration; Temp = growth temperature; mys = desired growth rate; OD = optical density at 600nm.

Figure 12 shows the scheme of lysis and purification of AG1Δ*ara*D S6wtd1EGFP/*ara*D2

Detailed description of the invention

The present invention is based on an effort to find an alternative, antibiotic-free selection system, which could be used in the production of recombinant therapeutic products to be administered *in vivo*, especially in the production of DNA vaccines. Surprisingly it was found that the *ara*D gene involved in the pentose phosphate pathway of both prokaryotic and eukaryotic organisms, such as mammalians including humans, can be successfully used as a selection marker in an auxotrophic host cell for the plasmid. The use of the auxotrophy has the advantage of not involving a use or generation of toxic substances that could later contaminate the plasmid preparation.

An efficient selection system has been constructed on the basis of *araD/araC* genes [Ariza, R. R., *et al.*, Carcinogenesis 14 (1993) 303-305]. However, this selection system has been used in the studies on the mechanisms of mutagenesis but not used before as a selection marker for plasmid maintenance. Ariza *et al.* used a strain where the *ara*C gene contains a termination codon and the *ara*D gene is inactivated. A product of the *sup*F gene, which codes for a suppressor tRNA, was introduced on the plasmid. In the presence of active suppressor tRNA, enzymatically active product from *ara*C was produced causing cell growth arrest (because *ara*D was inactive). This system allows to study the suppression of mutations by supF tRNA: in case *sup*F is inactivated by mutation, the cells can grow on arabinose. Therefore, this selection system is based on *ara*C gene and not on *ara*D gene. *ara*D was not introduced into a plasmid, nor was the system designed or characterized for plasmid production purposes.

The *araD* gene codes for an enzyme which is responsible for epimerization of ribulose-5-phosphate to xylulose-5-phosphate (Fig. 1) and therefore allows the use arabinose in the pentose phosphate pathway [Engelsberg, E., *et al.*, J. Bacteriol. 84: (1962) 137-146]. If *araD* is inactivated, ribulose-5-phosphate accumulates in the bacterial cell leading to growth arrest.

If the chromosomal copy of *araD* is inactivated in the host cell and an intact copy of the *araD* gene, a complementary sequence thereof, or a catalytically active fragment thereof is inserted into the plasmid, the growth advantage of the plasmid-containing cells in medium containing L-arabinose is achieved as a result from two effects. First, the plasmid-containing cells can

use arabinose as a carbon source, and second, the toxic ribulose-5-phosphate does not accumulate. This allows the use of rich growth media supplemented with arabinose. In rich media the *E. coli* cells grow fast and the plasmid yield is high. Inexpensive standard components of the bacterial growth media, such as yeast extract, can be used as an amino acid source. The traces of ribulose-5-phosphate that theoretically could contaminate the plasmid preparation are not a problem, when the preparation is administered *in vivo*, as ribulose-5-phosphate can be efficiently metabolized by human cells and is not toxic.

The selection system of the invention comprises 1) a vector carrying the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and 2) a specific bacterial strain deficient of the *ara*D gene. When the specific host deficient of the *ara*D gene is cultured in the presence of arabinose, the only surviving cells are those containing the vector, which contains the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof.

In the selection system of the invention any expression vector commonly used in the production of therapeutic products can be employed, whereby the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof is inserted into the vector using methods generally known in the art. In the present context, the *ara*D gene comprises the sequence identified by SEQ ID No. 1, a complementary sequence thereof, or a sequence hybridizable thereto. In the present context, the term "the complementary sequence of SEQ ID. No 1" has its conventional meaning and the term "a catalytically active fragment of the *ara*D gene" is any gene fragment coding a polypeptide or a protein capable of epimerization of L-ribulose-5-phosphate to D-xylulose-5-phosphate. In a specific embodiment of the invention the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof is inserted in the vector capable of a long-term maintenance and thereby capable of providing a stable expression of the desired antigen(s).

In a specifically preferred embodiment of the invention the vector used is an expression vector comprising:

(a) a DNA sequence encoding a nuclear-anchoring protein operatively linked to a heterologous promoter, said nuclear-anchoring protein comprising (i) a DNA binding domain which binds to a specific DNA sequence, and

(ii) a functional domain that binds to a nuclear component, or a functional equivalent thereof; and

(b) a multimerized DNA sequence forming a binding site for the nuclear anchoring protein, wherein said vector lacks a papilloma virus origin of replication, and

(c) the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof.

Such vectors have been described in detail in the international patent application WO02/090558, which is incorporated herein by reference.

Most preferably the vector used in the selection method of the present invention is an expression vector comprising:

(a) the E2 protein of Bovine Papilloma Virus type 1 (BPV), and

(b) multiple binding sites of the BPV E2 protein incorporated into the vector as a cluster, where the sites can be as head-to-tail structures or can be included into the vector by spaced positioning, wherein said vector lacks a papilloma virus origin of replication, and

(c) the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof.

In the selection system of the invention in principle any known host deficient of the *ara*D gene and suitable for use in the production of therapeutic products could be employed. In the present connection the term "deficient" denotes a host, in which the *ara*D gene is either totally deleted or inactivated by any known method.

In a preferred embodiment of the invention an *Escherichia coli* strain, preferably commercially available *E. coli* strains AG1 and JM109, from which the *ara*D gene has been deleted with generally known methods, such as those described below in the Examples, is used. Alternatively, commercially available *E. coli* strains AG1 and JM109, in which the *ara*D gene has been inactivated by any known method can be employed. In the method for selection of cells carrying a gene of interest for the production of recombinant therapeutic products, the gene of interest is inserted into the *ara*D deficient host cell using method well known in the art and the cells are cultured in a growth medium containing arabinose under culturing medium and conditions suitable the host in question.

Any growth medium suitable for culturing *E. coli* cells can be used. For commercial production the growth medium will naturally be optimized in

terms of the yield. Examples of suitable growth media are commercially available growth media, such as M9 and LB (available from several manufacturers, such as Fermentas, Lithuania). The amount of arabinose added in the growth medium is not critical but naturally arabinose should be present in an amount that is sufficient for the total culturing period. As low amount as 0.1% has been found sufficient for the selection. Typically arabinose is added to the medium in an amount of about 0.1% to about 2.0%, preferably in an amount of about 0.2% to about 1,0%, most preferably 0.2% to about 0.5%. However the effect of L-arabinose is observed at concentrations as low as 0.01% and L-arabinose can be added up to 5% in the growth medium. In a special embodiment, where L-arabinose is used both as a selecting agent and as a limited carbon source, 0.2% of L-arabinose is a suitable amount to be added into the growth medium.

The selection system of the invention is suitable for use in any expression system. It is especially suitable for use in the expression of recombinant therapeutic products, such as DNA vaccines, intended for use *in vivo,* since the problems associated with the use of antibiotic resistance genes are avoided. Likewise the selection system of the invention is suitable for use in the production of recombinant proteins.

The possible contamination of arabinose in the final product resulting from the preparation process is inconsequential, since arabinose is editable sugar contained in foods naturally and as an additive and thus not toxic to mammalians including humans.

Additionally, the araD gene is smaller in size than the commonly used antibiotic resistance genes against, for instance, ampicillin and tetracyclin and of similar size to kanamycin and chloramphenicol resistance genes. This affords an additional advantade, since allows the construction of small plasmids for which the energy consumption for replication is smaller than for large plasmids. Thereby both the growth rate of bacterial culture and plasmid yield are increased.

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

Example 1

Cloning of *ara*D selection plasmids

For cloning *araD* selection constructs plasmid S6wtd1EGFP (Figures 2) was used. It has pMB1 origin of replication and kanamycin resistance marker as functional elements of plasmid backbone. The kanamycin resistance in this plasmid is conferred by gene that is derived from *E.coli* transposon Tn903.

The *araD* gene was amplified using polymerase chain reaction (PCR) from *E. Coli DH5α* chromosome according to standard procedure. The PCR product was cloned into selected plasmids in two different orientations with the primer pairs s6*ara*DL1 + s6*ara*DR1 or s6*ara*DL1 + s6*ara*DR1, generating products named *ara*D1 and *ara*D2, respectively:

s6*ara*DL1:

CGCCATGGTTCTCATGTTTGACAGCTTATCATCGATAAGCTTTA ATGCGGTAGTTTAGCACGAAGGAGTCAACATG (SEQ Id. No. 2);

s6*ara*DR1:

CGCCATGGACTAGTAAAAAAAAGCCCGCTCATTAGGCGGGCT GTCATTACTGCCCGTAATATGC (SEQ Id. No. 3);

s6*ara*DL2:

CGCCATGGACTAGTTCTCATGTTTGACAGCTTATCATCGATAAG CTTTAATGCGGTAGTTTAGCACGAAGGAGTCAACATG (SEQ Id. No. 4);

s6*ara*DR2:

CGCCATGGAAAAAAAAGCCCGCTCATTAGGCGGGCTGTCAT-TACTGCCCGTAATATGC (SEQ Id. No. 5);

The primers were designed so that P2 promoter from plasmid pBR322 (used for driving the tetracycline resistance gene in pBR322) and termination sequence from *trp* operon of *E. coli* were added during PCR to the upstream and downstream of *ara*D coding sequence, respectively.

PCR products of 814 and 815 bp were cloned into pUC18 vector linearized with HincII (Fermentas, Lithuania) and correct sequences were verified by sequencing using universal sequencing primers

M13F22: GCCAGGGTTTTCCCAGTCACGA (SEQ Id. No. 6) and

M13R24: GAGCGGATAACAATTTCACACAGG (SEQ Id. No. 7) and

araD specific primers

*ara*D F311: CCAACTCACCGGCTGCTCTATC (SEQ Id. No. 8),

*ara*D F614: AATGCCGAAGATGCGGTGCATAAC (SEQ Id. No. 9),

*ara*D R700: TAACTGCGGCGCTAACTGAC (SEQ Id. No. 10), and

*ara*D R421: GGTTGCTGGAATCGACTGAC (SEQ Id. No. 11).

The mutations in amplified sequences were repaired by recombination of different clones.

For cloning *araD* into S6wtd1EGFP, the vector was linearized by partial digestion with restriction enzyme PagI (position 4761) (Fermentas, Lithuania) and DNA 5'-termini were dephosphorylated with Calf Intestine Alkaline Phosphatase (CIAP; Fermentas, Lithuania). *ara*D1 and *ara*D2 fragments were cut out from pUC18 with NcoI (Fermentas, Lithuania) and ligated to S6wtd1EGFP/PagI.

Both ligation mixtures were transformed into *E. coli DH5α* competent cells and plated onto dishes containing LB medium containing 50 µg/ml kanamycin and incubated at 37°C over night. Colonies were first analysed with colony PCR and whereafter the DNA was isolated and digested with different restriction enzymes.

The cloning resulted in plasmids S6wtd1EGFP*kana*/*ara*D1, S6wtd1EGFP*kana*/*ara*D2, which are shown in Figures 3 and 4

To remove the kanamycin resistance marker gene from the plasmids, S6wtd1EGFP*kana*/*ara*D1 and S6wtd1EGFP*kana*/*ara*D2 were digested with restriction endonuclease BcuI (Fermentas, Lithuania) and a 6473 bp vector fragment was self-ligated.

The ligation mixtures were transformed into an *E. coli AG1 ΔaraD* strain (see Example 2) and plated onto dishes containing M9 media supplemented 2% L-arabinose and incubated at 37°C for 36 hours. Colonies were first analysed with colony PCR and whereafter the DNA was isolated and digested with different restriction enzymes. The cloning resulted in plasmids S6wtd1EGFP/*ara*D1, S6wtd1EGFP/*ara*D2, respectively, are shown in Figures 5 and 6

The bacterial colonies containing S6wtd1EGFP/*ara*D1 and S6wtd1EGFP/*ara*D2 were grown in two different media: LB supplemented with 2.5% L-arabinose and M9 supplemented with 0.2% L-arabinose at 37°C with vigorous shaking. The cells were harvested and the plasmid DNA was extracted from the cell using QIAprep Spin Miniprep Kit (QIAGEN) and analysed by agarose gel electrophoresis (Figures 7A and 7B, respectively).

The plasmid DNA samples from cultures in LB and M9 media were analysed by agarose gel electrophoresis before and after digestion with restriction endonuclease PagI (Fermentas, Lithuania), (Figure 8). The predicted sizes of the fragments obtained in the PagI digestion were 3954 and 2519 bp for S6wtd1EGFP/*ara*D1 and 4315 and 2157 bp for S6wtd1EGFP/*ara*D2. Lambda DNA digested with Eco91I (M15 in Figure 8C) and lambda DNA digested with EcoRI/ HindIII (Fermentas, Lithuania) (M3 in Figure 8C) were used as molecular weight markers. All analysed clones were correct in the restriction enzyme analysis, but the DNA yield was very low when the plasmids were grown in LB media. Two of the analysed four S6wtd1EGFP/*ara*D2 clones (#13 and #14 in figure 8B) had higher copy number when grown in M9 media supplemented with 0,2% L-arabinose (Figures 7 and 8).

Example 2

Construction of arabinose sensitive Δ*araD Escherichia coli* strains.

Two *E .coli* strains, AG1 and JM109, were used to construct Δ*araD* mutants. The *araD* gene in *E. coli* genome was disrupted using the method described by Datsenko and Wanner [PNAS 97 (2000) 6640-6645]. This method exploits a phage λ Red recombination system. Briefly, the strategy of this system is to replace a chromosomal sequence with a selectable antibiotic resistance gene that is generated by PCR by using primers with homology extensions. This is accomplished by Red-mediated recombination in these flanking homologies.

For transformation of the pKD46 [Datsenko and Wanner, PNAS 97 (12) (2000)], which encodes the phage λ recombination system, into *E. coli* strains AG1 and JM109, these cells were made chemically competent using RF1 and RF2 solutions:

RF1 100ml

RbCl 1.2 g

$MnCl_2 \cdot 4H_2O$ 0.99 g

1 M KAc pH 7.5 3 ml
$CaCl_2 \cdot 2H_2O$ 0.15 g
Glycerol 15 g
pH 5.8 (add CH_3COOH)

RF2 100ml
0.5 M MOPS 2 ml
RbCl 0.12 g
$CaCl_2 \cdot 2H_2O$ 1.1 g
Glycerol 15 g
pH 6.8 (add NaOH)

The cells were grown in 2 ml of LB medium to OD_{600} 0.2-0.5. The culture was centrifuged and the pellet was resuspended in 1 ml of RF1. The mixture was kept on ice for 10 min and centrifuged. The pellet was suspended in 100 µl of RF2 and the suspension was kept on ice for 30-45 min. Approximately 50 ng of pKD43 was added and the cells were kept on ice for additional 30 min followed by heat shock of 5 min at 37°C. After incubation for 10 min on ice 900 µl of SOB medium was added to the transformed cells and the mixture was incubated at 37°C for one hour. Cells were plated on LB medium containing ampicillin (100 µg/ml). The colonies were picked from the transformation plates and grown in 2 ml of the same medium to OD_{600} of approximately 1 and glycerol stocks were made (2 ml culture + 0.6 ml 50% glycerol). The stocks were stored at -80°C.

For disruption of the *araD* gene a linear PCR product which contains kanamycin resistance gene was generated. Plasmid pKD13 (Datsenko and Wanner, PNAS vol. 97, no 12, June 2000) was used as the PCR template. Primers used were *ara*(pr1) and *ara*(pr4):

***ara*(pr1)**
5'-CTCAAACGCCCAGGTATTAGAAGCCAACCTGGCGCTGCC-AAAACACGTGTAG GCTGGAGCTGCTTC 3' (SEQ Id. No 12)

***ara*(pr4)**
5'-GGTTTGATCACAAAGACGCCGCGCTCGCGATCAACGGCGC-ATTCCGGGGAT CCGTCGACC 3' (SEQ Id. No. 13)

These primers have the complement sequences with pKD13 for annealing in PCR and with the *ara*D gene for homologous recombination.

The PCR reaction mixture was as follows: PFU native buffer (5 μl), 10 mM dNTP (5 μl), primer *ara*(pr1) 10 μM (1 μl), primer *ara*(pr4) 10 μM (1 μl), pKD13 100 ng (2 μl), DMSO (4 μl), PFU 2.5 U (1 μl), and mQ water up to 50 μl.

The PCR procedure was as follows: denaturation 45 s, 96°C, annealing 45 s, 50°C, synthesis 2 min 30 s, 72°C, 25 cycles. The PCR product obtained was 1.4 kb.

Five reactions were performed simultaneously, the DNA was purified from 2% agarose gel using Ultrapure purification Kit (MoBio Labotratories Inc.) and eluted with 60 μl of water. The DNA was concentrated with ethanol precipitation and dissolved in 5 μl of water. The final concentration was 0.6 μg/μl. An aliquot of 1.5 μl was used in one electroporation.

The PCR product was electroporated into AG1 pKD46 (Datsenko and Wanner, supra) and JM109 pKD46 *E. coli* cells. First, 200 ml of YENB medium containing 10 mM of L-arabinose for the induction of the recombination system and 100 μg/ml ampicillin was inoculated with an overnight culture of AG1 pKD46 and JM 109 pKD46. The cultures were grown at 30°C to OD_{600} 0.8 (JM109) and 0.6 (AG1). The bacteria was collected by centrifugation at 4,000 g for 10 min at 4°C, washed twice with 20 ml of sterile water and once with 20 ml of sterile water containing 10% glycerol. The cells were suspended in 300 μl water containing 10% glycerol. Forty μl of competent cells were used in one electroporation.

The electroporation was performed with BioRad *E. coli* Pulser using 0.2 cm cuvettes and 2.5 kV voltage. The purified PCR product (1.5 μl) was added to the competent cells, kept on ice for 1 min, and immediately after the electroporation, 2 ml of warm SOB medium was added to the cells and the mixture was incubated at 37°C for 1 hour. The cells were plated on LB medium containg kanamycin (25 μg/ml). 100 pg of large kanamycin resistant plasmid (GTU-MultiHIV C-clade) was used as a positive control, no plasmid was added to the negative control. The transformation efficiency was 10^6 for AG1 and 10^7 for JM109 for positive control. There were no colonies on the negative control plate, 215 colonies were obtained on JM109+PCR product plate and 70 colonies on AG1+PCR product plate

Example 3

Testing of the *E. coli* AG1Δ*ara*D and JM109Δ*ara*D strains.

The colonies obtained from the electroporation as described in Example 2 were tested for the presence of kanamycin resistance gene by colony PCR using primers *ara*VlisF (5' CGGCACGAAGGAGTCAACAT 3'; SEQ Id. No. 14) and *ara*VlisR (5' TGATAGAGCAGCCGGTGAGT 3'; SEQ Id. No. 15) which contain annealing sites on the *ara*D gene near the insertion site. A PCR product of 272 bp was expected from the *E. coli* AG1 and JM109 strains without insertion in *ara*D and a 1545 bp product, if the PCR product had been inserted in the *ara*D gene. Nine colonies of AG1Δ*ara*D and 14 colonies of JM109Δ*ara*D out of 15 were checked and gave the 1545 bp product. It was therefore concluded that these strains contained the kanamycin resistance gene insertion.

To confirm the insertion of kanamycin gene another colony PCR was performed using primers *kana*SF (5'TCAGATCCTTGGCGGCAAGA3'; Sequence Id. No 16) and *ara*VR (5'TGTAATCGACGCCGGAAGGT3'; Sequence Id. No. 17). These primers produce a 435 bp product, if the kanamycin resistance gene has been inserted into the *ara*D gene. Six colonies from both strains were tested and all gave the correct product.

Six colonies of AG1Δ*ara*D and 6 colonies of JM109Δ*ara*D were plated on LB medium containing 25 μg/ml of kanamycin and incubated at 37°C overnight to eliminate the pKD46 plasmid, which has a temperature sensitive replication origin. The cells were tested for ampicillin sensitivity by replica plating on LB medium and LB medium containing ampicillin. None grew on the medium containing ampicillin and it was concluded that the bacteria does not contain the pKD46 plasmid any more.

Arabinose sensitivity was tested on the produced AG1Δ*ara*D and JM109Δ*ara*D strains. One colony of AG1Δ*ara*D and one colony of JM109Δ*ara*D were each inoculated into 2 ml LB. The cultures were grown for 8 hours, diluted 1:100 into M9 medium containing 0.2% glycerol, 25 μg/ml kanamycin, 0.01% thiamine (0.05% proline for JM109Δ*ara*D) and different concentrations of L-arabinose were added in the growth medium. The cultures were grown overnight at 37°C in shaker incubator and OD_{600} was measured (Table 1).

Table 1. Testing of arabinose sensitivity.

L-arabinose %	AG1Δ*ara*D OD_{600}	JM109Δ*ara*D OD_{600}
0	3.2	1.9
0.1	0.03	0.03
0.2	0.030	0.026
0.5	0.030	0.020
1	0.024	0.025
2	0.017	0.021

As seen in Table 1 as low amount as 0.1% of L-arabinose is enough to inhibit the growth of the Δ*ara*D strains of the invention

Additionally the plasmid DNA yield of Δ*ara*D strains was tested.

Plasmid S6wtd1EGFP*ara*D2 prepared in Example 1 was transformed into AG1Δ*ara*D and JM109Δ*ara*D strains. Competent cells were prepared with RF1 and RF2 solutions as described in Example 2.

The colonies from the transformation plates were inoculated into 2 ml of M9 medium containing 0.5% yeast extract and 25 μg/ml kanamycin + 0.01% thiamine + L-arabinose (2% and 0.2%).

The cultures were incubated at 37°C for 17 hours. Then the OD_{600} was measured to quantitate the cell density and the plasmid DNA was extracted with Qiagen Miniprep Kit. Coefficient 2.8 (OD_{600}/ml) was used for miniprep isolation to get comparable results. The results are shown in Table 2.

DNA concentration was mesured with sepctrophotometer as OD at 260 nm. For microscopic analysis a drop of bacterial culture was layed on glass slide and covered with cover slip. The culture was looked at with 100x objective in oil immersion.

Table 2. Plasmid DNA yield of Δ*ara*D strains

Strain	L-arabinose (%)	OD_{600}	Plasmid DNA conc. (μg/μl)	Plasmid DNA yield (μg per ml of culture)	Appearance in microscope
AG1Δ*ara*D	2	7.6	0.039	5.3	no filaments
AG1Δ*ara*D	0.2	5.8	0.057	5.9	no filaments
JM109Δ*ara*D	2	4.9	0.043	3.8	very few filaments
JM109Δ*ara*D	0.2	4.3	0.038	2.9	very few filaments

According to these results 0.2% L-arabinose is sufficient for obtaining the plasmid copy number at the same level as with 2% arabinose.

For this plasmid AG1Δ*ara*D seems to be better because the plasmid yield is somewhat higher and cell densities also.

Example 4

Stability of S6wtd1EGFP/*ara*D2

An important feature of the vaccination vector is the stability during propagation in bacterial cells. To test the stability of S6wtd1EGFP/*ara*D2 in bacteria the plasmid was transformed into the *E. coli* AG1Δ*ara*D and JM109Δ*ara*D strains prepared in Example 2 and the intactness of the vector was followed by the plasmid DNA analysis during four generations.

The plasmid S6wtd1EGFP/*ara*D2 was mixed with competent *E. coli* AG1Δ*ara*D and JM109Δ*ara*D cells and incubated on ice for 30 minutes. Subsequently, the cell suspension was subjected to a heat-shock for 3 minutes at 37°C followed by a rapid cooling on ice. One milliliter of LB medium was added to the sample and the mixture was incubated for 45 minutes at 37°C with vigorous shaking. Finally, a portion of the cells was plated onto M9 medium dishes containing 0.5% yeast extract, 2% L-arabinose and 25 μg/ml of kanamycin. On the next day, the cells from one colony were transferred onto the new dish containing the same medium. This procedure was repeated until four passages of bacteria had been grown. Two colonies from each passage of both bacterial strains were used to inoculate of 2 ml of M9 medium containing 0.5% yeast extract, 2% L-arabinose and 25 μg/ml of kanamycin incubated overnight at 37°C with vigorous shaking. The cells were harvested and the plasmid DNA was extracted from the bacteria using QIAprep Spin Miniprep Kit (QIAGEN). The plasmid DNA samples before (Figure 9.) and after the digestion with restriction endonuclease HindIII (Figure 10) (Fermentas, Lithuania) were analyzed by agarose gel electrophoresis in comparison with the original S6wtd1EGFP/*ara*D2 DNA used for transformation (as control in Figures 9 and 10). Lambda DNA digested with EcoRI/HindIII (Fermentas, Lithuania) was used as a molecular weight marker (M3 in Figure 10).

Samples were digested with HindIII as shown in figure 10A for *E.coli* AG1Δ*ara*D and in Figure 10B for JM109Δ*ara*D strain, patterns identical to the original S6wtd1EGFP/*ara*D2 plasmid DNA were observed. The predicted sizes of the fragments resulted by HindIII digestion are 3274, 1688 and 1510 bp. It can be concluded that the vaccination vector S6wtd1EGFP/*ara*D2 is stable when propagated in *E. coli* AG1Δ*ara*D and JM109Δ*ara*D strains.

Example 5

Fed-batch fermentation of AG1Δ*ara*D S6wtd1EGFP/*ara*D2

The araD gene based selection system was also tested in fed-batch fermentation for the purpose of production of plasmid containing bacteria A single colony was picked from AG1Δ*ara*D S6wtd1EGFP/*ara*D2 plate and inoculated into 250 ml M9 medium containing 0.5% yeast extract, 0.2% L-arabinose and 25 μg/ml of kanamycin and incubated overnight at 37°C with vigorous shaking. After 18 hours the OD_{600} of inoculum was 6.4. 160 ml of inoculum was added to fermenter containing 5 l Fermenter Starting Medium (8 g/l KH_2PO_4; 10 g/l NaCl; 5 g/l NH_4Cl; 5 g/l yeast extract; 2 g/l L-arabinose; 2 g/l $MgSO_4$, 25 mg/l kanamycin and 0.1 g/l thiamine; pH 6.7 with NH_4OH). After 5.5 hours of growth automatic feeding was started with given growth speed of 0.15 h^{-1} (allows carbon-source limited growth) with fermenter feeding medium (300 g/l L-arabinose; 150 g/l yeast extract; 50 mg/l kanamycin; 0.2 g/l thiamine). Feeding speed was controlled by computer according to formulae $F(t)=myS*S_{in}/S_f$ where myS is desired growth rate, S_{in} is the amount of carbon source added to the time point and S_f is carbon source concentration in feeding medium. Growth was followed by measuring OD_{600} and samples for plasmid DNA were taken. The data registered during fermentation is represented in Figure 11. Fermentation was terminated when 1 l of feeding medium was consumed. Final OD_{600} was 45. The bacterial mass was collected by centrifugation and washed once with 2 l STE buffer. Yield of bacterial biomass was 410 g wet weight. The data for plasmid DNA content is shown in table 3.

Table 3.

Plasmid DNA yield during AG1Δ*ara*D S6wtd1EGFP/*ara*D2 fermentation

Time	OD_{600}	Plasmid DNA conc. (μg/μl)	Plasmid DNA yield (μg per ml of culture)
Inoculum	6.4	0.04	4.6
4 h	3.1	0.02	1.1
21 h	28	0.1	50
24 h	37	0.13	87
29 h	45	0.14	113

The data in Table 3 indicate that the L-arabinose selection system works very well at high cell densities. It is probably because more plasmid copies in bacterial cell gives an advantage in the conditions of L-arabinose limitation by enabling the bacterium to use sugar more rapidly.

Example 6

Purification of AG1Δ*ara*D S6wtd1EGFP/*ara*D2

The purification of AG1Δ*ara*D S6wtd1EGFP/*ara*D2 was performed as follows (Figure 12):

a) Feeding preparation

Clear lysate was prepared according to Qiagen's Plasmid Purification Handbook, exept RNase was not used.

200g of E. coli cell paste was resuspended in 2000ml of Resuspension Buffer and later equal volumes of P2 and P3 for lysis and neutralization were used. The cell debris was removed by centrifugation at 6000g for 30 minutes at 4°C. Clear lysate was poured through the paper towel, 1/10 of 10% Triton X-114 (Sigma) was added and solution was left on ice for 1 hour. (Triton X-114 has been shown to effectively reduce the level of endotoxins in protein, Liu et al., Clinical Biochemistry, 1997) After one hour nucleic acids were precipitated with 0,6 volumes of cold isopropanol. Supernatant was decanted and precipitate was stored overnight at –20°C.

b) Plasmid DNA purification

Plasmid DNA purification was performed according to Amersham Pharmacia's three step supercoiled plasmid purification process, where few modifications were adopted.

Step 1. Precipitate was redissolved in 1500ml TE (10mM Tris-Cl, 1mM EDTA; pH 8.0) and loaded for RNA removal and buffer exchange on Sepharose 6 FF (Amersham Pharmacia), previously equilibrated with Buffer A – 2M $(NH_4)_2SO_4$, 100mM Tris Cl, 10mM EDTA, pH 7.5.

Step 2. Void volume was directed to the PlasmidSelect (Amersham Pharmacia) column (equilibrated with Buffer A) and after washing and elution with Buffer B2 (1,6M NaCl, 2M $(NH_4)_2SO_4$, 100mM Tris Cl, 10mM EDTA, pH 7.5), supercoiled plasmid DNA was captured.

Step 3. Eluted plasmid was diluted with five volumes of distilled, deionized water and loaded to SOURCE 30Q (Amersham Pharmacia) equilibrated with buffer C1 (0,4M NaCl, 100mM Tris Cl, 10mM EDTA, pH 7.5) . After washing, purified plasmid was eluted with Buffer C2 (1M NaCl, 100mM Tris Cl, 10mM EDTA, pH 7.5) and elution peak was collected. Fraction size was 150ml and it contained 100mg of endotoxins-free (<10 EU/mg) S6wtd1EGFP/*ara*D2 plasmid.

L2

Claims

1. A selection system comprising a vector carrying the *ara*D gene or L-ribulose-5-phosphate 4-epimerase gene (EC 5.1.3.4.), a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and a bacterial cell deficient of the *ara*D gene.

2. A selection system according to claim 1, wherein the bacterial cell is an *Escherichia coli* cell.

3. A selection system according to claim 2, wherein the *E. coli* is a *E. coli* strain JM109.

4. A vector comprising the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker.

5. A vector according to claim 4, wherein the vector is an expression vector comprising:

(a) a DNA sequence encoding a nuclear-anchoring protein operatively linked to a heterologous promoter, said nuclear-anchoring protein comprising (i) a DNA binding domain which binds to a specific DNA sequence, and (ii) a functional domain that binds to a nuclear component, or a functional equivalent thereof; and

(b) a multimerized DNA sequence forming a binding site for the nuclear anchoring protein, wherein said vector lacks a papilloma virus origin of replication, and

c) the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker.

6. A vector according to claim 5, wherein the vector is an expression vector comprising:

(a) a DNA sequence encoding a nuclear-anchoring protein operatively linked to a heterologous promoter, wherein the nuclear-anchoring protein is the E2 protein of Bovine Papilloma Virus type 1 (BPV), and

(b) a multimerized DNA sequence forming a binding site for the nuclear anchoring protein is of multiple binding sites the BPV E2 protein incorporated into the vector as a cluster, where the sites can be as head-to-tail structures or can be included into the vector by spaced positioning, wherein said vector lacks a papilloma virus origin of replication, and

c) the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker.

7. *E. coli* strain AG1 deficient of the *ara*D gene.

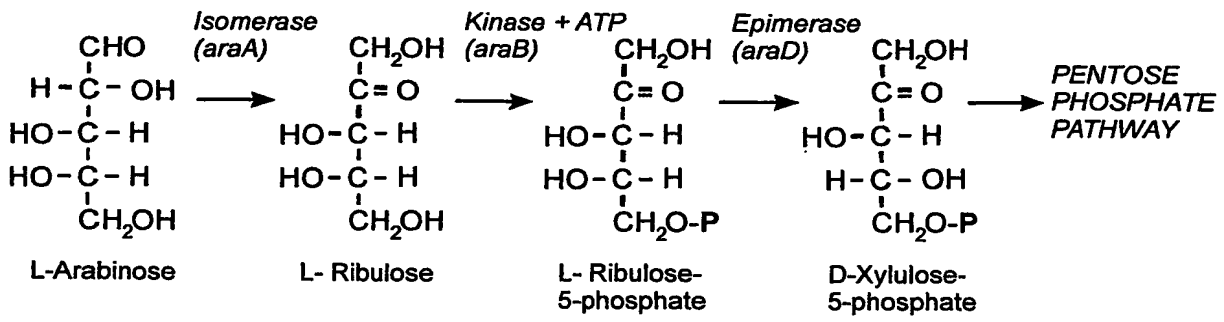
8. *E. coli* strain JM109 deficient of the *ara*D gene.

9. A method of selecting the cells transformed with a plasmid containing the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and the gene of interest, the method comprising inserting the plasmid into the *ara*D deficient host cell and growing the cells in a growth medium containing arabinose.

L 3

(57) Abstact

The present invention relates to a novel selection system, which is based on the use of the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof as a selection marker and to the use of a bacterial strain deficient of the *ara*D gene. The present invention further relates to novel vectors containing the *ara*D gene, a complementary sequence thereof, or a catalytically active fragment thereof and to novel bacterial strains deficient of the *ara*D gene. The present invention additionally relates to a method of selecting the cells transformed with a plasmid, which contains the gene of interest.



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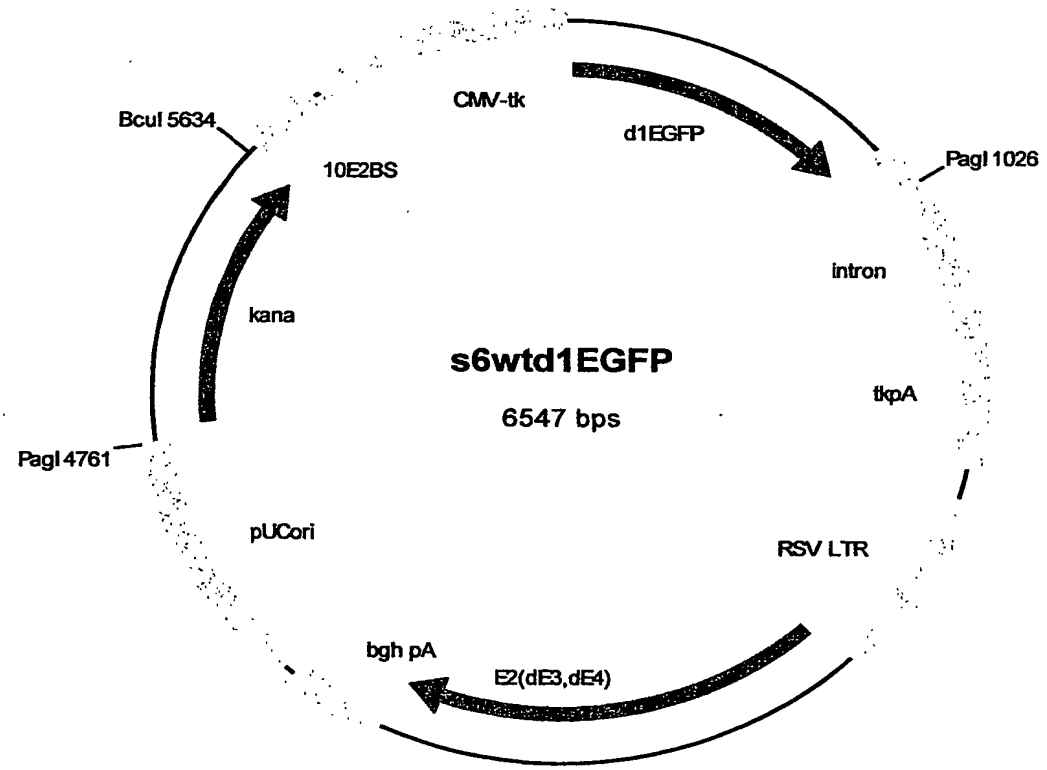

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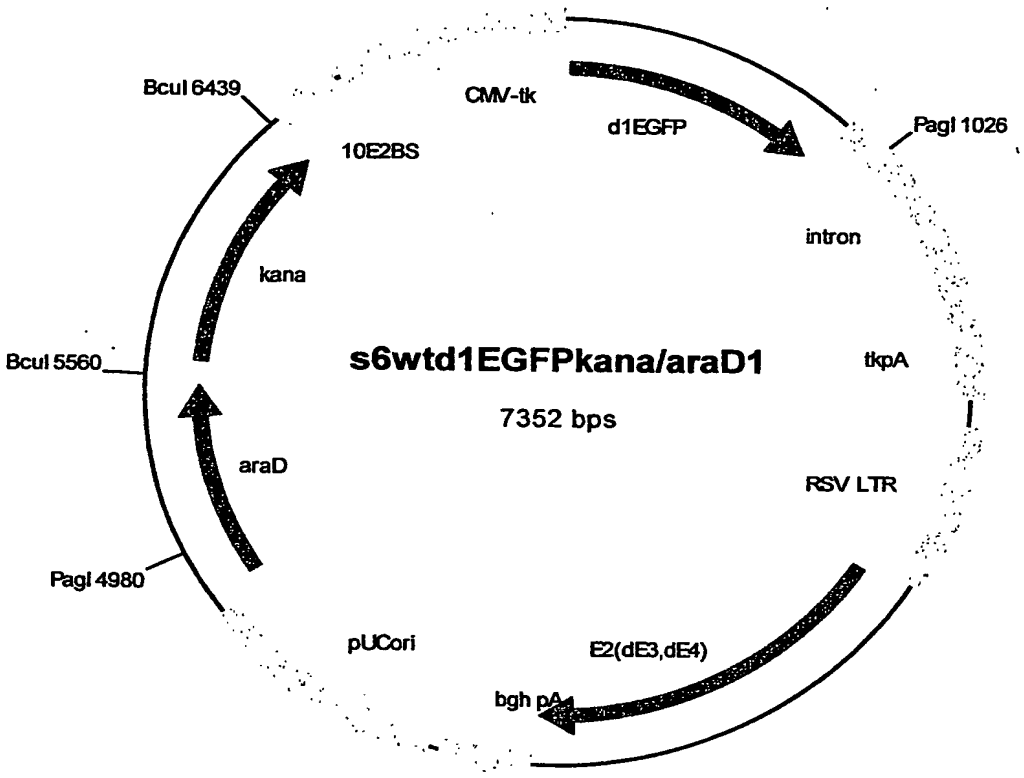
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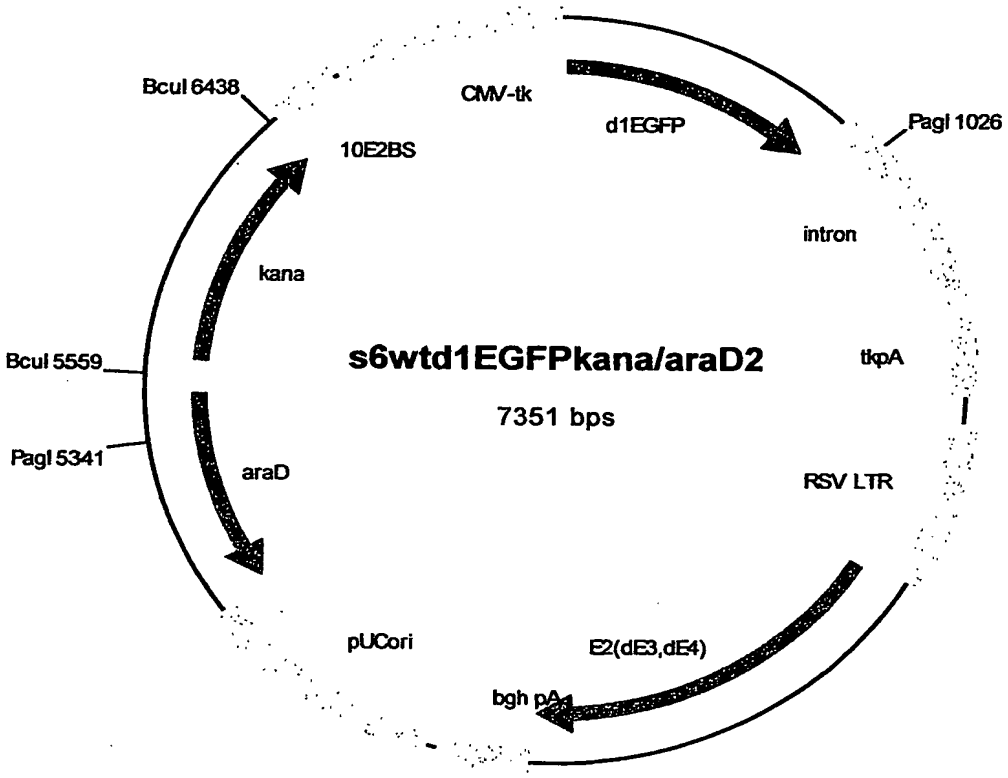
Figure 3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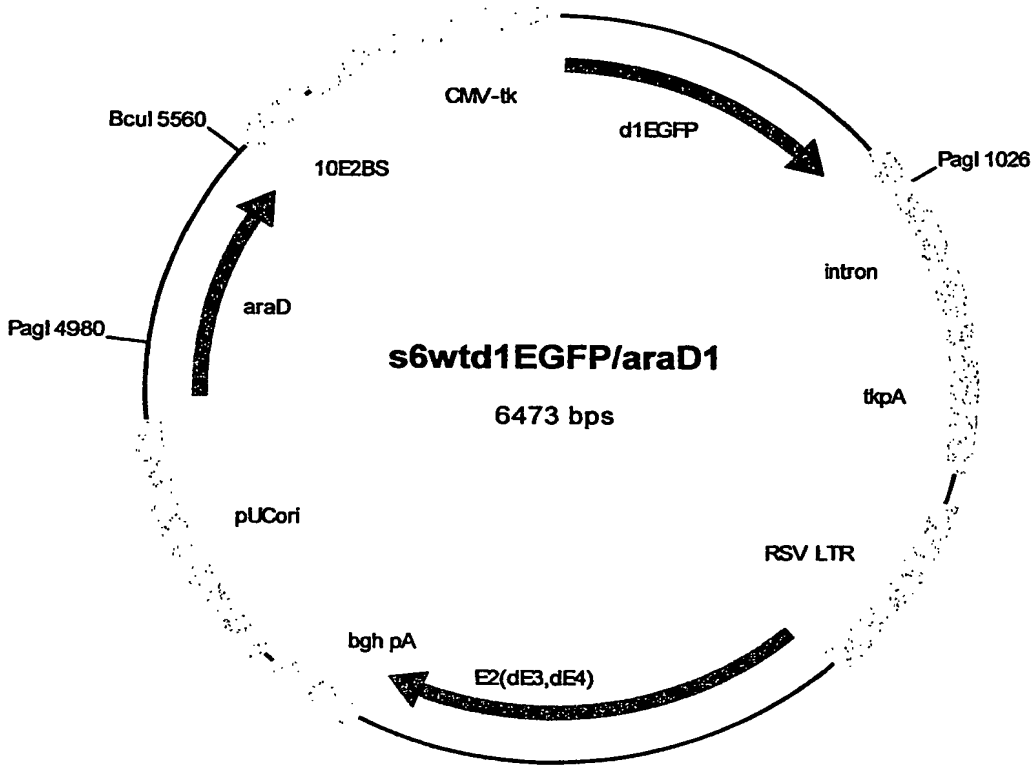
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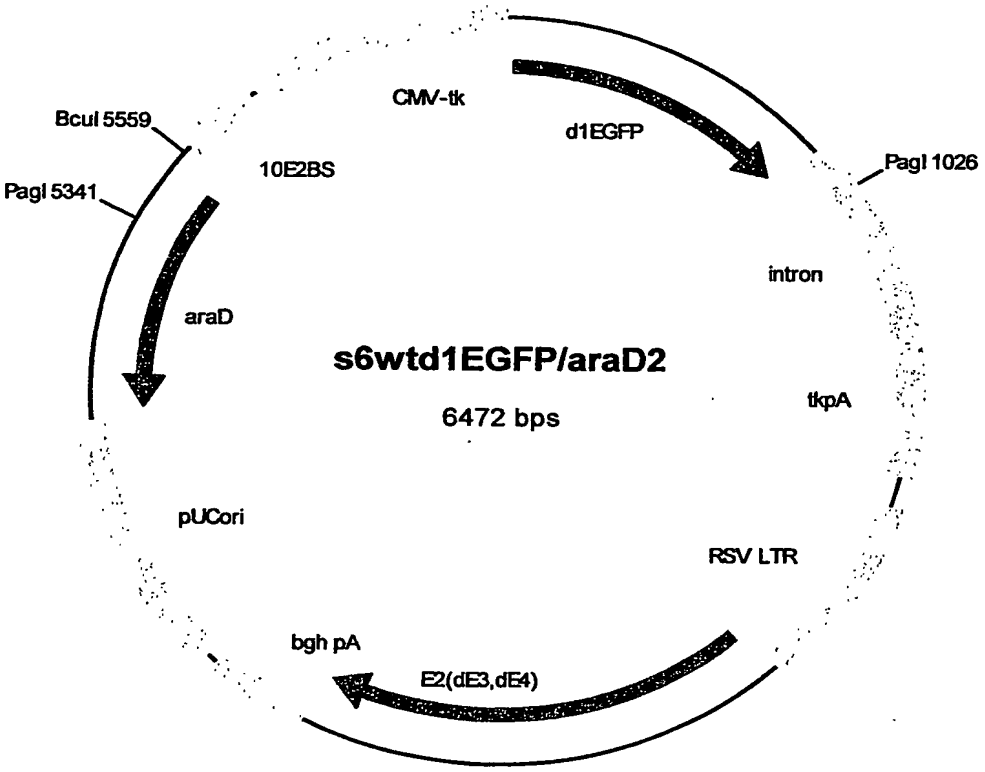
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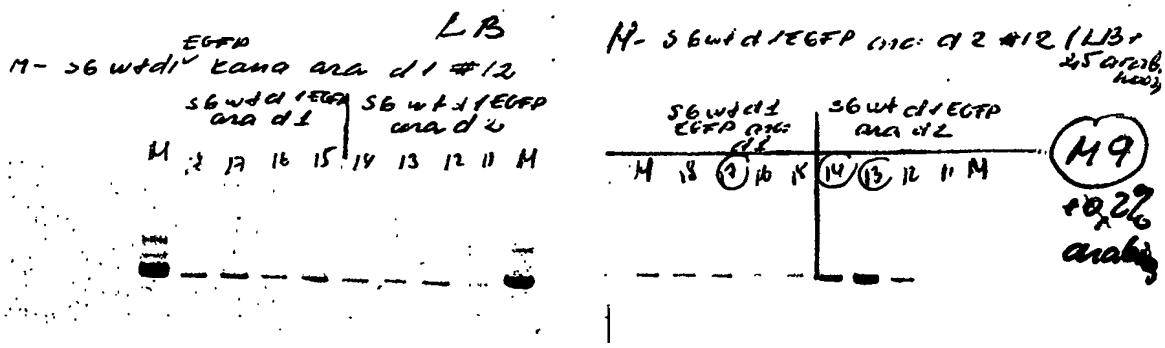
Figure 6

7A 7B



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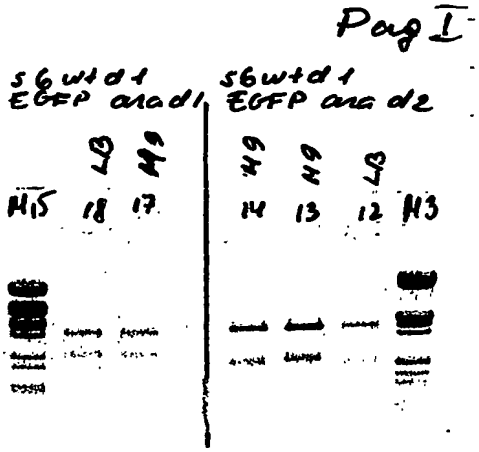

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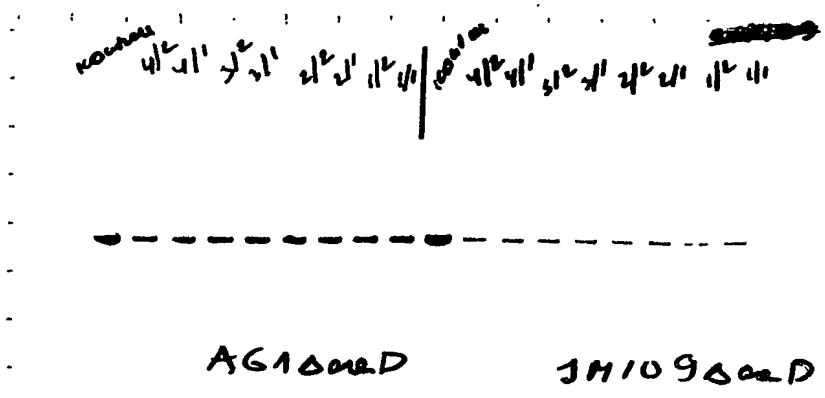

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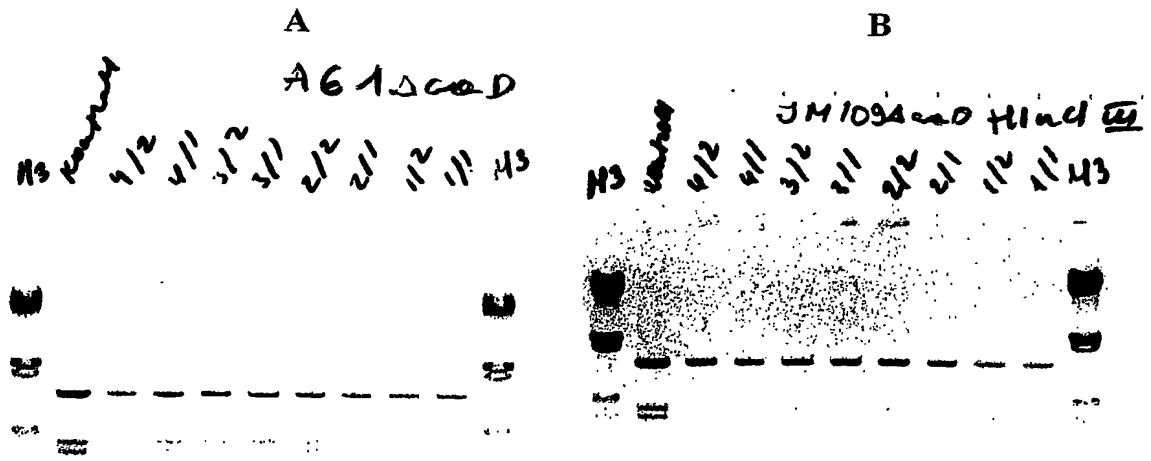
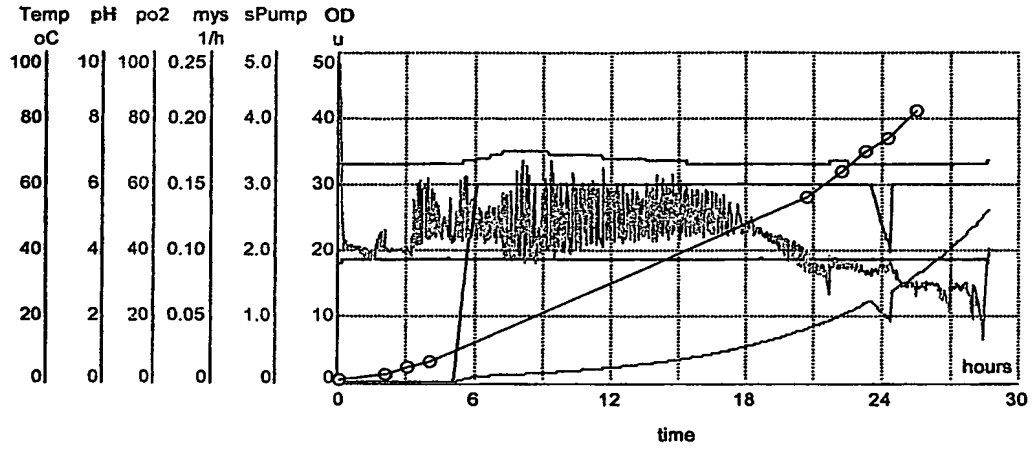


Figure 10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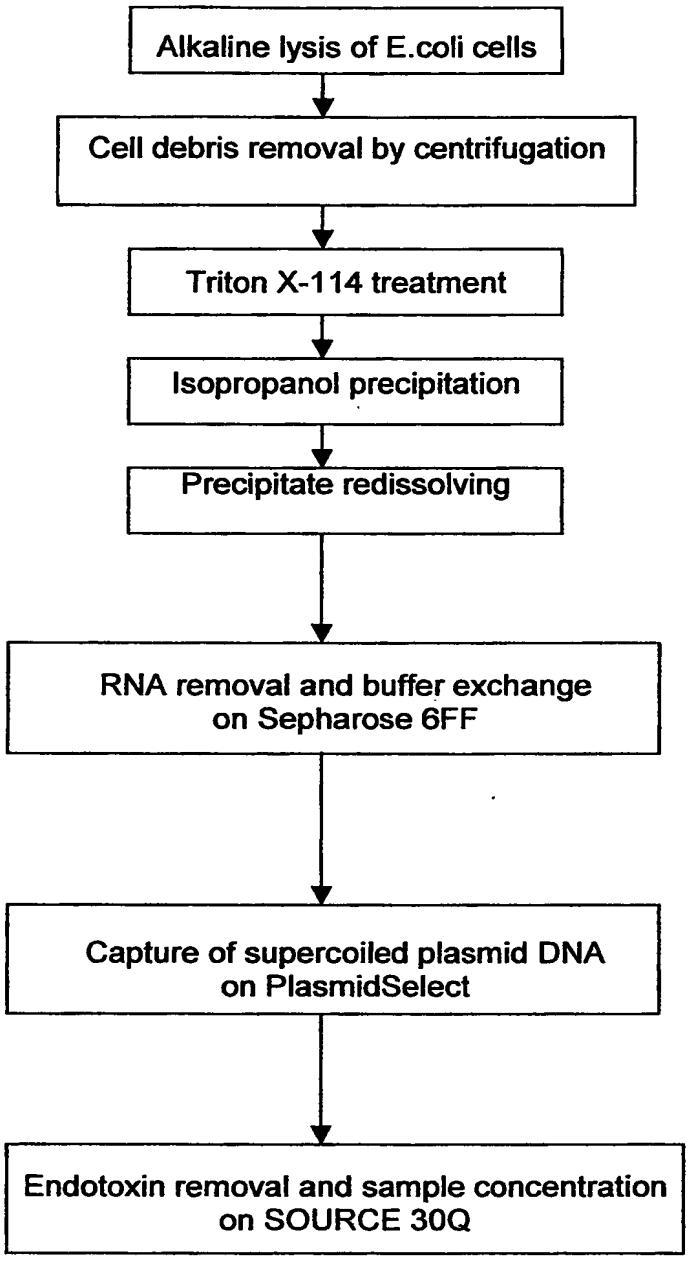
time

Figure 11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Cell debris removal by centrifugation
Triton X-114 treatment
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RNA removal and buffer exchange on Sepharose 6FF
Capture of supercoiled plasmid DNA on PlasmidSelect
Endotoxin removal and sample concentration on SOURCE 30Q

Figure 12

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